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The Role of Reactive Oxygen Species in Oxidative-Induced Neoplastic Transformation

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Abstract

The generation of reactive oxygen species (ROS) is a normal occurrence in the life of a cell. ROS are derived from both exogenous and endogenous sources and take part in a plethora of normal physiological mechanisms from host immunity to cell cycle regulation. However, ROS also exhibit a deleterious, disruptive character that means they must now be taken seriously as a genuine carcinogenic agent able to alter a variety of pathways leading to the initiation of cancer. The two-sidedness of ROS means that the relationship between ROS and the onset and progression of cancer is hard to determine. ROS and reactive nitrogen species (RNS) are able to initiate cancer through the damage of DNA leading to the genetic instability that drives the early stages of cancer. Simultaneously, they can activate a number of signalling cascades and transcription factors that facilitate uncontrolled cell growth. The production of ROS in neoplastic cells can lead to the production of new blood vessels that provide the machinery for subsequent metastasis and tumour invasion. It is important that the role of ROS in these pathways are fully elucidated in order to provide potential therapies measures.

Introduction

The toxicity of oxygen in biological systems is an unfortunate paradox given that much of life is dependent on the molecule for survival. It is only recently that the profound implications of the oxygen paradox have come to the fore, as has the role that oxidative stress plays in cellular damage and disruption. Oxidative stress was first demonstrated to damage living organisms in 1952 when Conger and Fairchild demonstrated the presence of chromosomal aberrations in pollen grains due to increased oxygen pressure [1]. Since then, many destructive functions of oxygen and its derivatives have been elucidated with human cancer, amongst other diseases such as atherosclerosis, at the centre of investigation. It is now widely accepted that oxidative DNA damage is a predominant factor in the aetiology of human cancer and reactive oxygen species (ROS) have been found to induce many forms of DNA damage that can often lead to genetic instability and the initiation of cancerous growth [2]. ROS and reactive nitrogen species (RNS) have been shown to participate in a plethora of factors that lead to neoplastic transformation, including deregulation of intracellular signalling pathways and cell cycle control. This review will outline the major pathways affected by free radicals and how this dysregulation

can ultimately lead to malignant cancerous growth. Attention will first be drawn to the key oxidative players in neoplastic transformation and how the action of ROS and RNS can promote neoplastic transformation.

Reactive Oxygen Species

Free radicals are chemical species that possess one or more unpaired electrons and are able to react with each other to produce new radical species, which can result in a chain reaction of radical formation [3]. However, ROS refers to both the radical and non-radical derivatives of oxygen. As molecular oxygen is abundant in normal cellular conditions, free radical propagation involving reactive oxygen species is a common place event within the cell. Common free oxygen radicals include hydroxyl radical ($^{\bullet}OH$), nitric oxide ($^{\bullet}NO$), superoxide ($O_2^{\bullet-}$) and singlet oxygen [2].

ROS in Neoplastic Transformation

Under normal redox conditions ROS have been implicated in initiation of apoptosis, cell cycle arrest and cellular senescence. In apoptopic processes, ROS generation is attributed to the uncoupling of the electron transport chain and to a decrease in mitochondrial membrane potential. Furthermore, oxidation of mitochondrial membrane proteins by ROS may contribute to cytochrome c release due to further disruption of mitochondrial membrane potential However, under oxidative stress ROS can act in stark contrast by stimulating carcinogenesis [4]. Cancer development is a multistage process requiring the cumulative action of multiple deleterious events that occur in one cell clone. These events include a three stage model which include a permanent change in the genetic material of one somatic cell (initiation); the expansion of the mutated cell clone (promotion); and the malignant conversion into cancer (progression) (see Valko *et al*., 2006 [6]). ROS play a prominent role in all three stages of cell transformation and act to interfere in a number of crucial pathways including DNA repair, signal transduction, angiogenesis, apoptopic inhibition and metastasis [5].

Tumour Initiation

ROS induced DNA damage

For initiation of carcinogenesis, a permanent genetic alteration must occur in a given cell that will ultimately be inherited by the progeny of the cell. However, the DNA modification must be sufficiently tenacious as to escape efficient repair mechanisms, but not so excessive that apoptosis ensues [7]. A persistent overproduction of ROS can lead to consecutive DNA lesions that can lead to cell transformation, as shown by the presence of oxidative modifications of DNA in malignant cancer cells [8]. In particular, production of hydroxyl radical in proximity to DNA generates a whole series of DNA damage by a variety of mechanisms. Chemical alteration of deoxyribose elements by the hydroxyl radical leads to the release of purine and pyrimidine bases, producing abasic sites that have shown to be mutagenic *in vivo*. Furthermore, hydroxyl radical attack of DNA generates radical adducts within adenine and guanine residues that results in ring-fragmented bases such as the 5-formamido-4,6-diamino-pyrmidine and 8-hydroxy-guanine (8-OH-Gua) [9]. Due to its lowest oxidation potential guanine is the most easily oxidised among the four DNA bases. Hydroxyl radical can attack C-4, C-5, and C-8 of a guanine nucleobase generating the radicals $G4OH^{\bullet}$, $G5OH^{\bullet}$ and $G8OH^{\bullet}$ respectively. The radical adducts $G4OH^{\bullet}$ and $G5OH^{\bullet}$ can eliminate a molecule of water giving $G(-H)^{\bullet}$ radical which, by gaining an electron and protonation, is transformed back to guanine. However, the

adduct G8OH$^{\bullet}$ subsequently produces two forms of DNA damage: reduction of the adduct's form in which the radical is localised on N-7 gives hemi-aminal which opens the imidazole ring forming 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPyG). Alternatively, oxidation of the C-4 and C-2 resonance forms of G8OH$^{\bullet}$ followed by the loss of a proton yields 8-OH-Gua [10].

8-OH-Gua is the most ubiquitous oxidative DNA base lesion occurring in approximately one in 10^5 guanine residues in a normal cell and therefore, the most widely studied lesion. 8-OH-Gua is able to induce GC → TA transversions and mutagenesis unless repaired prior to DNA replication [5]. GC → TA transversions are frequently detected in the RAS oncogene and represent a possible mechanism of tumour initiation by ROS [13]. Moreover, recent studies have suggested a positive correlation between tumour size and 8-OH-Gua concentration, offering itself as a potential biomarker in the early stages of tumour initiation and promotion [14]. Several other ROS modified bases have also been shown to possess miscoding potential and pre-mutagenic properties including 2-hydroxyadenine, 8-hydroxyadenine, 5-hydroxyctosine and 5-hydroxyuracil. Whereas the hydroxyl radical is almost indiscriminate in its attack upon DNA, singlet oxygen is much more selective. It is inefficient at producing strand breakage and generates mainly guanine-derived products including 8-OH-Gua and FAPyG, although also common in hydroxyl-mediated DNA damage [13]. DNA polymerase is known to be sensitive to damage induced errors at guanine residues and the contribution of oxidative damage to polymerase-specific 'hotspots' is a major contributor to DNA polymerase-mediated mutagenesis [14]. It should be noted that DNA damage caused by oxidative stress need not necessarily involve direct attack on DNA by ROS and RNS [15]. ROS are able to influence intracellular free calcium (Ca^{2+}) levels by depleting endogenous antioxidant stores, providing an important signal leading to Ca^{2+} mobilisation. The result of ROS-mediated Ca^{2+} changes is the activation of endonucleases leading to DNA fragmentation [5]. For the most part, many of the ROS induced oxidative damage will be successfully repaired by DNA repair mechanisms. It has been estimated that 2×10^4 DNA damaging events occur per cell per day and a large proportion of this figure is ROS induced [3] and therefore, ROS species play a major part in introducing genetic instability within the cell, especially under oxidative stress. This genetic instability is merely the first step in neoplastic transformation that can ultimately lead to tumour promotion in the progeny.

Tumour Promotion

In addition to the ROS-mediated mutagenesis involved in initiation and cancer progression, oxidative stress can stimulate the expansion of mutated cell clones by modulating genes related to cell proliferation and cell death. While high levels of oxidative stress inhibits proliferation via cytotoxic effects, intermediate levels can stimulate cell division and therefore, promote tumour growth (fig 1.). This characteristic relationship between free radical levels and tumour promotion may provide an explanation as to why higher tolerance to oxidative stress, due to increased antioxidant levels, can lead to a promotable phenotype [15]. ROS have been observed to influence a number of intracellular signalling pathways that leads to subsequent activation of transcription factors associated with cell proliferation and inhibition of cell death.

Oxidative Stress, Cell signalling and Transcription Factors

As mentioned above, the effect of ROS in defining cell fate is concentration dependent. At low concentrations, superoxide and hydrogen peroxide in fact stimulate cell proliferation and enhanced cell survival by acting as secondary messengers in a number of signalling cascades. This section will review the major signalling pathways influenced by ROS and how their subsequent stimulation can lead to the activation of transcription factors involved in cell proliferation [6].

Non-receptor Tyrosine Kinases

Several non-receptor protein kinases (PTKs) belonging to the Src family and Janus kinase (JAK) are also activated by ROS. Hydrogen peroxide and superoxide radicals are able to induce tyrosine phosphorylation of several PTKs in various cell types including fibroblasts, T and B lymphocytes, macrophages and myeloid cells. Over expression of Src has been associated with colorectal, breast, pancreas, bladder and head/neck cancers. Activated Src binds to cell membranes by myristilation and initiates mitogen-activated protein kinases (MAPKs), NF-κB and phosphoinositide 3-kinase (PI3K) signalling pathways resulting in cell proliferation [16].

Protein Tyrosine Phosphatases

Protein tyrosine phosphatases (PTPs) are arguably the best characterised direct targets of ROS. Reversible inhibition of PTPs by ROS plays an essential role in redox control and cell signalling and furthermore, inactivation of PTPs can directly trigger protein tyrosine kinase activation. The effects of ROS occur through targeting the cysteine-containing residues of the active sites of tyrosine phosphatases as cysteine residues are highly susceptible to oxidative damage, particularly through hydrogen peroxide. The reaction generates sulphenic acid intermediates that further react with thiols to form catalytically inactive PTP disulfides. Thus, not only are ROS involved in the excessive stimulation of activatory pathways, they initiate a double blow to the cell by knocking out inhibitory mechanisms mediated by protein tyrosine phosphatases [16][17].

Serine/threonine Kinases

The ability of free radicals to induce large increases in cytosolic Ca^{2+} levels has a knock on effect for many serine/threonine kinases and ultimately the transcription factors they in turn activate. The induction of proto-oncogene c-FOS by low levels of ROS was found to be directly mediated by cytosolic Ca^{2+} [18]. The activation of protein kinase C (PKC) by ROS also mediated in part by Ca^{2+}, leading to the phosphorylation and activation S6-kinase which is involved in the acquisition of growth competence [19]. PKC contains several cysteine-rich regions both in the zinc finger of the regulatory domain and in the catalytic site, which can be modified by various ROS. Both regulatory and catalytic domains of PKC are susceptible to hydrogen peroxide-induced modification, often leading to its activation. PKC is thought to mediate many of the ROS induced cell proliferation events [6]. A number of serine and threonine kinases of the mitogen-activated protein kinase (MAPK) family can also be regulated as well as activated by ROS. The role of MAPKs in carcinogenesis is a complicated story in view that activation of many MAPK pathways is tissue and stimuli specific. There are four known MAPK families: extracellular-regulated (ERKs), c-jun-NH_2-terminal kinase (JNK), p38 MAPK and the big MAPK-1 (BMAPK-1). Products of NAD(P)H oxidase 1 (NOX1) activity, superoxide and hydrogen peroxide, have been shown to activate MAPK cascades at the level of MEK and ERK1/2 [16]. However, it has also been reported that

endogenous hydrogen peroxide production by the respiratory burst induces ERK but not p38 kinase activity [20]. On the other hand, exogenous hydrogen peroxide preferentially activates p38 but not ERK in rat alveolar macrophages [16]. Furthermore, p38α is able to initiate apoptosis in response to oncogene-induced ROS accumulation and plays a key role in the regulation of malignant transformation although, interestingly, highly tumourigenic human cancer cells can override this aspect of p38α function and therefore, promote cell survival [21]. This example highlights the complex nature of the relationship between ROS and signal transduction pathways. It must be noted that the association is not only cell specific but concentration dependent. Often, under normal physiological conditions, ROS act to inhibit NF-κB rather than activate it, thereby initiating apoptosis and inhibiting cell proliferation. It is the oxidation of cysteine residues in the DNA binding domain of the p50 subunit by ROS that affects the DNA-binding capacity of NF-κB. This complex involvement of ROS is carried forward upon activation of transcription factors.

NF-κB

NF-κB is a redox regulated transcription factor and a sensor for oxidative stress and plays a major role in cell proliferation [22]. Composed primarily of a heterodimer consisting of the subunits p50 (NF-κB1) and p65 (relA). Transcription factors of the NF-κB/c-Rel family can be directly activated by application of oxidising agents, especially hydrogen peroxide, although hypoxia is also able to induce nuclear translocation of NF-κB and NF-κB activation [23]. Storz et al. [24] demonstrated an important role for the serine/threonine kinase PKD in the activation of NF-κB via the IκB kinase (IKK) complex in cells exposed to hydrogen peroxide [24]. The IKK complex is thought to play a prominent role in the ROS-mediated activation of NF-κB. Oxidation of cysteine179 of IKKα and IKKβ binds IKK with other components of the signalosome maintaining them in a more stable conformation. Furthermore, it has been postulated that certain components of the signalosome are maintained in a non-functional form by a redox sensitive inhibitor such as thioredoxin, which has been shown to inhibit members of the MAP3K family. Therefore, ROS-dependent activation of MAP3K family members is a result of potential inhibitors like thioredoxin, leading to subsequent NF-κB activation [25][26]. NF-κB activation pathways associated with cancer are uncoupled from their normal modes of regulation and in cancer cells a constitutive NF-κB activation has been illustrated. For example, BCR-Abl transformed cells show constitutive activation of NF-κB and Hodgkin disease has been associated with a mutation in the gene which encodes IκBα [26]. NF-κB has been implicated in the development of leukemia and lymphoma as well as a host of carcinomas including breast, gastric and colorectal. ROS-induced NF-kB activation can also stimulate many of the genetic alterations required to render cells tumourigenic including the activation of growth stimulatory targets such as interleukin-2 (IL-2), granulocyte macrophage-colony stimulating factor (GM-CSF), CD40L; regulation of the cell cycle through G1 cyclins and c-Myc and inhibit apoptosis [15]. Consequently, NF-κB plays a prominent role in the progression of neoplastic cells. Although its pleiotropic effects are often complex and yet to be fully understood, further investigation can provide possibilities for potential development of therapeutic strategies.

AP-1 and HIF-1

AP-1 is an assembly of dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun, Fos, Maf, and ATF subfamilies. AP-1 activity is initiated on activation of MAPK signalling pathways and like NF-κB, plays a key role in cell

proliferation. Activation of the transcription factor by hydrogen peroxide is mediated by JNK and p38 MAPK cascades and subsequent ROS-mediated inhibition of MAPK phosphatases also leads to increased MAPK activation and therefore, increased AP-1 expression. ROS are also known to promote the expression of c-Fos and c-Jun which act as positive regulators of cell proliferation as well as function as positive or negative regulators of apoptosis [6] Hydrogen peroxide, in addition to nitric oxide, has also been shown to up regulate the expression of hypoxia-inducible factor 1 (HIF-1), a heterodimer consisting of HIF-1α and HIF-1β (arylhydrocarbon receptor-nuclear translocator [ARNT]) [22]. While ARNT is constitutively expressed, it is the HIF-1α subunit that carries the sensitivity towards molecular oxygen. HIF-1 regulates the expression of a variety of genes expressed in neoplastic cells such as vascular endothelial growth factor (VEGF), which plays a key role in angiogenesis, aldolase, enolase and lactate dehydrogenase. HIF-1, as well as being expressed under hypoxic conditions, is induced by the expression of oncogenes Src and Ras and therefore HIF-1 is over expressed in many cancers [28].

p53

The inactivation and loss of tumour suppressor genes marks a critical point in the progression of neoplastic transformation. At this junction the mechanisms that inhibit cell growth after DNA damage has incurred cease to function and cell proliferation is left to take place unregulated and indefinitely [7]. p53 is a prominent tumour suppressor gene with over 70% of cancers bearing defects in p53 function. p53 controls cell cycle via a multitude of molecular pathways and is able to induce apoptosis through transcriptional regulation of pro-apoptopic proteins. A study by Souici et al [29] found that p53 exposure to nitric oxide leads to GC → TA transitions in the first base of codon 248 of the p53 gene. This mutation results in an amino acid change abolishing the wild-type function of the protein supporting the notion that nitric oxide is a potent carcinogenic agent, particularly under inflammatory conditions. A positive correlation has been observed between GC → TA transitions at CpG dinucleotide sites in the p53 gene and inducible nitric oxide synthase activity [30]. The likelihood of nitric oxide induced mutation of p53 appears to increase at sites of chronic inflammation where there is a continual production of nitric oxide by nitric oxide synthase. High mutation rates in a number of p53 gene codons have been observed in inflamed lesions of the colon with patients with ulcerative colitis [31]. Elevated GC → AT transitional mutations have also been observed in lymphoid, oesophageal, head, stomach, brain and breast cancers which also demonstrate increased iNOS levels [32] However, under many normal physiological conditions, ROS promote increased expression of p53 leading to greater cell cycle arrest and in many instances, cell senescence [33]. So, as seems to be the recurring theme with ROS induced neoplastic transformation, the picture is far from clear. Increased ROS levels actually lead to an accumulation of p53 in the cell so once again an important concentration dependent mechanism is in place. It is firmly established the nitric oxide can induce p53 mutation but more work needs to be done to investigate the cell specific, ROS specific, and concentration relationship between various other important ROS in p53 dysfunction.

Tumour Progression

The final stage of cancer development is the acquisition of malignant properties by the tumour including accelerated growth; escape of immune surveillance; angiogenesis; and metastasis. Most of these changes require further formation of DNA lesions and through elevated ROS levels, tumour cells remain in a consistent

state of oxidative stress that can increase genomic instability and metastatic potential [15]. Conversely, many tumours exhibit regions of hypoxia which results in the activation of signalling pathways leading to increased cell proliferation and angiogenesis. However, the presence of hypoxia in malignant tumours is its self a response to inadequate rates of angiogenesis [34], hinting at the presence of a feedback mechanism at work in malignant cells. The adaptation of cancerous cells to survive under hypoxia is a key feature of transformed cells that contributes significantly to their survival and aggressive phenotypes. The reoxygenation of blood and the reperfusion of hypoxic tissue can increase the concentration of ROS. ROS produced via this reoxygeneration have many damaging effects but can also stimulate angiogenesis which constitutes one of the most significant events in tumour progression [35]. Blood flow within newly generated vessels is often chaotic which leads to further bouts of hypoxia, followed by subsequent reperfusion events causing the release of additional ROS [4]. ROS can induce the production of angiogenic factors VEGF and interleukin-8 (IL-8) and can promote the secretion of matrix metalloprotease-1 (MMP-1) which also promotes vessel growth within the tumour microenvironment. Furthermore, oxidative stress can trigger vasodilation on activation of heme oxygenase-1, which generates carbon monoxide and iNOS which generates nitric oxide. Both can act as vasodilators. Generation of new blood vessels increases the risk of blood born metastasis that can considerably increase a tumour's invasiveness [4]. When certain neoplastic cells are under oxidative stress they exhibit decreased attachment to the basal lamina and thus, are more able to detach themselves from basement membranes and enter the vasculature [36]. ROS regulate cell adhesion by modulating integrin function and integrin-induced signalling pathways in which Rac1 is thought to play a prominent role by altering cell to cell and cell to matrix adhesion, influencing cancer cell motility and invasion. Rac1 activity is in turn able to induce ROS production in endothelial cells and functions in ROS-mediated actin cytoskeleton rearrangement [4].

Conclusion

The generation of ROS is a consequence of aerobic life and is unavoidable. ROS represent a constant source of assault upon DNA, which promotes genetic instability that can lead to the initiation of cancerous growth [6]. It is now firmly established that oxidative stress plays a key role in all stages of cancer that begins with the altering of DNA and ends with malignancy and tumour invasion. ROS can influence many of the signal transduction pathways that mediate cell proliferation and apoptosis that can eventually lead to uncontrolled neoplastic growth. In tumour promotion, the production of ROS can modulate neovascularisation, an important step in the potential onset of metastasis. However, the relationship between ROS and the onset of cancer is far from being clearly defined. In many cases increased levels of ROS can protect against cancer by inducing apoptosis, the immune response to transformed cells is often to increase levels of oxidative stress to initiate cytotoxic mechanisms in the host cell. Therefore, it appears that intermediate to low levels of ROS results in tumour initiation [37]. Particularly in the case of carcinomas, hypoxia actually drives cancer progression through the onset of angiogenesis. This complicated picture has especially drastic consequences for cancer therapy. If high levels of ROS can protect against cell proliferation, then therapeutics in the form of antioxidants that target ROS to be scavenged can increase tumour initiation. It is for that reason that new research must now also focus on the role of antioxidants in chemoprevention in order to characterise accurately their potential therapeutic roles

in a range of tissues and cancers. Antioxidants gained from dietary intake are a key focal point as much is made of their potential to fight against illnesses including cancer and atherosclerosis. However, investigations so far have produced much conflicting evidence. Dietary components such as green tea are often consumed in the hope that they can provide vital antioxidants that can protect against oxidative-induced transformation. It is imperative then, that we fully understand the antioxidant composition of such foods so that we can apply that knowledge to potential therapies in targeting reactive oxygen species in neoplastic transformation.

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